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DATE: March 25, 2004

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FROM: Kelly J. Williamson

Re: U.S. Patent Application for *Targeted Manipulation of Genes in Plants*
Appl. No. 09/579,784; Filed May 26, 2000
Client Ref. 0804D2
Our File 5718-23B (035718/199392)

Attachments:
Supplemental Information Disclosure Statement(2 pages)
PTO Form 1449(1 page)
3 citations (104 pages)

Part II of IV

NO. OF PAGES: ~~108~~ 35
(Including cover page)

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REQUESTED BY: Pam Lockley
VOICE NUMBER:

RTA01/2110098v1

(19) Europäisches Patentamt
European Patent Office
Office européen des brevets

(11) Publication number: **0 492 113 A2**

(12) EUROPEAN PATENT APPLICATION

(21) Application number: 91119264.0

(22) Date of filing: 12.11.91

(51) Int. Cl.5: C12N 15/60, C12N 9/88, C12N 15/82, A01N 63/00, A01H 1/04

(30) Priority: 27.12.90 US 633210

(43) Date of publication of application: 01.07.92 Bulletin 92/27

(84) Designated Contracting States: AT BE CH DE DK ES FR GB GR IT LI LU NL SE

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(54) Herbicide resistant AHAS deletion mutants.

(57) This invention provides novel nucleic acid sequences encoding herbicide-resistant AHAS enzymes. These sequences contain deletions of one or more amino acids in "conserved" regions of the AHAS molecule. Also disclosed are vectors containing novel sequences, as well as herbicide-resistant plants and plant cells transformed thereby.

EP 0 492 113 A2

This invention relates to novel DNA sequences that encode novel variant forms of acetohydroxy acid synthase enzyme (hereinafter AHAS). The AHAS enzyme is a critical enzyme routinely produced in a variety of plants and a broad range of microorganisms. Normal AHAS function is inhibited by a number of different types of herbicides; however, new AHAS enzymes encoded by the mutant DNA sequences function normally catalytically even in the presence of such herbicides and, therefore, confer herbicide resistance upon the plant or microorganism containing them.

The novel DNA sequences are based on the unexpected observation that deletion of one or more specific amino acids in certain regions of the normal AHAS gene sequence results in a fully functional enzyme, but renders the enzyme resistant to inhibition by a variety of different types of herbicides, including imidazolinones, triazolopyrimidines, and sulfonylureas. The availability of these variant sequences provides a tool for transformation of a variety of different crop plants to herbicide resistance, as well as providing novel selectable markers for use in other types of genetic transformation experiments.

BACKGROUND OF THE INVENTION

The use of herbicides in agriculture is now widespread. Although there are a large number of available compounds which effectively destroy weeds, not all herbicides are capable of selectively targeting the undesirable plants over crop plants, as well as being non-toxic to animals. Often, it is necessary to settle for compounds which are simply less toxic to crop plants than to weeds. In order to overcome this problem, development of herbicide resistant crop plants has become a major focus of agricultural research.

An important aspect of development of herbicide-resistance is an understanding of how the herbicide works in inhibiting plant growth, and then manipulating the affected biochemical pathway in the crop plant so that the inhibitory effect is avoided while the plant retains normal biological function. One of the first discoveries of the biochemical mechanism of herbicides related to a series of structurally unrelated herbicide compounds, the imidazolinones, the sulfonylureas and the triazolopyrimidines. It is now known (Shanor et al. Plant Physiol. 76: 545-546,1984. U.S. Patent No. 4,761,373) that each of these herbicides inhibits plant growth by interference with an integral cellular enzyme, acetohydroxyacid synthase (AHAS; also referred to as acetolacetate synthase, or ALS). AHAS is required for the synthesis of the amino acids isoleucine, leucine and valine.

The AHAS enzyme is known to be present throughout higher plants, as well as being found in a variety of microorganisms, such as the yeast Saccharomyces cerevisiae, and the enteric bacteria, Escherichia coli and Salmonella typhimurium. The genetic basis for the production of normal AHAS in a number of these species has also been well characterized. For example, in both E. coli and S. typhimurium three isozymes of AHAS exist; two of these are sensitive to herbicides while a third is not. Each of these isozymes possesses one large and one small protein subunit; and map to the IlvIH, IlvGM and IlvBN operons. In yeast, the single AHAS isozyme has been mapped to the ILV2 locus. In each case, sensitive and resistant forms have been identified and sequences of the various alleles have been determined (Friden et. al., Nucl. Acid Res. 13: 3979-3993, 1985; Lawther et al., PNAS USA 78: 922-928, 1982; Squires et al., Nucl. Acids Res 611: 5299-5313, 1983; Wek et al; Nucl. Acids Res 13: 4011-4027, 1985; Falco and Dumas, Genetics 109, 21-35, 985; Falco et al, Nucl. Acids Res 13: 4011-4027, 1985).

In tobacco, AHAS function is encoded by two unlinked genes, SuRA and SuRB. There is substantial identity between the two genes, both at the nucleotide level and amino acid level in the mature protein, although the N-terminal, putative transit region differs more substantially (Lee et al, EMBO J. 7: 1241-1248, 1988). Arabidopsis, on the other hand, has a single AHAS gene, which has also been completely sequenced. Comparisons among sequences of the AHAS genes in higher plants indicates a high level of conservation of certain regions of the sequence; specifically, there are at least 10 regions of sequence conservation. It has previously been assumed that these conserved regions are critical to the function of the enzyme, and that retention of that function is dependent upon substantial sequence conservation.

It has been recently reported (EP 0257993) that mutants exhibiting herbicide resistance possess mutations in at least one amino acid in one or more of these conserved regions. In particular, substitution of certain amino acids for the wild type amino acid at these specific sites in the AHAS sequence have been shown to be tolerated, and indeed result in herbicide resistance of the plant possessing this mutation, while retaining catalytic function. These mutations have been shown to occur at both the SuRA and SuRB loci in tobacco; similar mutations have been isolated in Arabidopsis and yeast.

It has now been unexpectedly discovered that deletions of one or more amino acids within one or more of these "conserved" regions result not only in a functional AHAS enzyme, but also result in herbicide resistance. Sequence conservation normally implies that any change in these regions would not be tolerated and would therefore result in a nonfunctional protein. However, in the present case, it is particularly

surprising that enzyme function is retained, in view of the fact that the deletions not only eliminate an amino acid residue that is a structural component of the enzyme, but also necessarily result in residue shifting, thereby destroying the apparent conservation of the sequence containing the mutation. Thus, novel nucleic acid sequences are provided which are useful in transforming herbicide sensitive plants to herbicide resistant ones. The plants transformed therefore provide the basis for development of novel herbicide resistant plant varieties.

SUMMARY OF THE INVENTION

The present invention provides novel nucleic acid sequences encoding functional AHAS enzymes insensitive to a variety of herbicides. The sequences in question comprise the deletion of one or more codons encoding a specific amino acid within one or more designated so-called conserved regions in the wild type AHAS molecule. The identity of these sites, and the deletable codons will be discussed in greater detail below. The altered DNA sequences are useful in methods for producing herbicide resistant plant cells, said methods comprising transforming a target plant cell with one or more of the altered sequences provided herein. Alternatively, mutagenesis is utilized to create deletion mutants in plant cells or seeds containing a nucleic acid sequence encoding a herbicide sensitive AHAS. Such plant cells are then passed through tissue culture in order to regenerate plants which possess the herbicide resistant or insensitive trait. The invention thus also encompasses plant cells, plant tissue cultures, adult plants, and plant seeds that possess the deletion mutant nucleic acid sequences and which express functional, herbicide-resistant AHAS enzymes.

The availability of these novel herbicide resistant plants enables new methods of growing crop plants in the presence of herbicides. Instead of growing non-resistant plants, fields may be planted with the resistant plants of the present invention and the field routinely treated with herbicide, with no resulting damage to crop plants. Preferred herbicides for this purpose are imidazolinones, sulfonylureas, and triazolopyrimidines.

The mutant nucleic acids of the present invention also provide novel selectable markers for use in transformation experiments. The nucleic acid sequence encoding a resistant AHAS is linked to a second gene prior to transfer to a host cell, and the entire construct transformed into the host. Putative transformed cells are then grown in culture in the presence of inhibitory amounts of herbicide; surviving cells will have successfully acquired the second gene of interest.

The following definitions should be understood to apply throughout the specification and claims. A "functional" or "normal" AHAS enzyme is one which is capable of catalyzing the first step in the pathway for synthesis of the essential amino acids isoleucine, leucine and valine. A "conserved" sequence or region of AHAS is a series of nucleic acids or amino acids within the AHAS nucleic acid or amino acid sequence which is the same in at least two of the species having the AHAS enzyme. A "wild-type" AHAS sequence is a sequence present in a herbicide sensitive member of a given species. A "resistant" plant is one which produces a normal AHAS enzyme, and which is capable of reaching maturity when grown in the presence of normally inhibitory levels of herbicide. The term "resistant", as used herein, is also intended to encompass "tolerant" plants, i.e., those plants which pheno-typically evidence adverse, but not lethal, reactions to the herbicide.

BRIEF DESCRIPTION OF THE FIGURES

Figures 1a and 1b (Sequence Listings 1 and 2) show, respectively, the amino acid and nucleotide sequences of Arabidopsis wild-type AHAS. In Figure 1a, boxed regions indicate exemplary subsequences in which deletions may be made to produce herbicide resistance. Circled residues identify specific sites where such deletions may be made. In Figure 1b, an arrow indicates the start of the coding region.

Figure 2(a-c) shows three photographs of progeny (first generation), resulting from a transgenic tobacco plant (10-1) transformed with a construct containing a Trp574 deletion mutation of Arabidopsis AHAS sprayed post-emergence with Pursuit® Imidazolinone herbicide at 0, 10, 20, 40, 60, 80 and 160 grams/hectare (g/ha). All three photographs were taken 25 days after spraying. In all photographs, control plants (a wild-type herbicide sensitive tobacco cultivar, Wisconsin 38[W38]) are in the front row.

(a) **W38 Control and 10-1 Self** - W38 represents the control and 10-1 the selfed transgenic tobacco progeny. At 0 g/ha Pursuit®, both the W38 and the 10-1 look similar. At 10 g/ha, the W38 plant is slightly inhibited in its growth as compared to 10-1; however, starting at 20 g/ha, W38 growth is almost completely inhibited. At 80 g/ha, 10-1 plant growth appears to be slightly inhibited and at 160 g/ha, 10-1 plant growth is clearly inhibited.

(b) **W38 Control and 0 x 10-1** - W38 as previously described. 0 x 10-1 represents W38 as the maternal